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ORIGINAL ARTICLE

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Circadian and developmental regulation of vacuolar invertase expression in petioles of sugar beet plants

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Abstract The expression pattern of the genes coding for vacuolar and extracellular invertase activity was analyzed in sugar beet (*Beta vulgaris*) and compared with the expression of sucrose synthase in this important sucrose-storing crop. Northern blot analysis revealed that sucrose synthase is the predominant sucrose-cleaving enzyme in tap roots, whereas vacuolar invertase was specifically expressed in petioles. Extracellular invertase transcripts showed low abundance in all the sugar beet organs and were not detected in northern blots. Relative RT-PCR analysis revealed differential expression of the two extracellular invertase genes: BVInv-CW1 was almost exclusively expressed in tap roots and BVInv-CW2 was widely expressed in all the organs analyzed. A remarkable result of this analysis was the high expression of vacuolar invertase (BVInv-V3) in petioles. Two factors had a clear influence on vacuolar invertase gene expression in petioles: light and the developmental stage, so that expression was higher in petioles from juvenile plants. BVInv-V3 transcripts showed circadian oscillation in petioles, with maximal accumulation during the light period. A similar pattern of diurnal oscillation was also observed for the vacuolar invertase activity, showing a delay with respect to the level of transcripts. The analysis of sugars in petioles revealed oscillation of the hexoses, with a remarkably higher content of glucose than fructose. In contrast, the level of sucrose in petioles was very low. This pattern of expression suggests an important role of petiole vacuolar invertase in plant development and photoassimilate partitioning.

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Keywords Circadian regulation · Acid invertase · Petiole · Sucrose synthase · Tap root

Abbreviations RACE: Rapid amplification of cDNA ends · RT-PCR: Reverse transcriptase-polymerase chain reaction · BVInv-V: *Beta vulgaris* vacuolar invertase · BVInv-CW: *Beta vulgaris* cell wall invertase · SuSy: Sucrose synthase · UTR: untranslated region

Introduction

Sucrose is an important molecule for the distribution of photoassimilates and serves as a source of carbon and energy to maintain cell metabolism and plant growth (Sturm and Tang 1999). There is increasing evidence of the function of sucrose (Chiou and Bush 1998) and glucose, as a signal for assimilate partitioning, exerting a relevant role in the regulation of genes involved in photosynthesis or carbohydrate metabolism (Koch 1996, 2004; Sheen et al. 1999; Smeekens 2000; Gibson 2000). In addition, sugar-responsive mutants show defects in phytohormone response or metabolism (Finkelstein and Gibson 2001; Rolland et al. 2002), thus revealing a close relationship of sugar metabolism and plant growth and development. Phytohormones, such as cytokinins, as well as environmental stimuli, play an important role in the regulation of source–sink relation; this function is mediated by the cytokinin regulation of sucrose hydrolytic enzymes (Roitsch 1999; Roitsch and Ehness 2000). Finally, the circadian clock seems to play an important role in the regulation of sugar partitioning between source and sink organs, allowing the plants to "anticipate" daily changes (Harmer et al. 2000; Rolland et al. 2002).

Sucrose utilization in sink tissues depends on its cleavage into hexoses, which is carried out by two different enzymes in plants, sucrose synthase and invertases (Tymowska-Lalanne and Kreis 1998; Sturm and Tang

1999; Winter and Huber 2000). Three types of invertases exist in plants, which differ in their biochemical properties, cell localization and function (Roitsch and González 2004). Neutral invertases are localized in the cytosol and serve catabolic purposes, whereas acid invertases are either bound to the cell wall, involved in sucrose partitioning and signal transduction, or localized in the vacuole, playing an important role in sugar storage, osmoregulation and abiotic stress response (Sturm 1999; Roitsch and González 2004). Genes encoding the enzymes of sucrose metabolism have been cloned from different plants. The analysis of these genes has shown that their expression is associated with the developmental stage of the plant, which has led to the invertase/sucrose synthase hypothesis for transitions of prominent stages of plant development (Wobus and Weber 1999; Koch 2004).

Sugar beet is a sucrose-storing crop with a concentration of sucrose in the tap root of up to 18% (Elliot and Weston 1993), where the sucrose is accumulated in the vacuole (Leigh et al. 1979). Previous studies have shown that soluble acid invertase activity in tap root, corresponding to a vacuolar isoenzyme (Leigh et al. 1979), decreased prior to sucrose storage, whereas storage of sucrose was coincident with increased sucrose synthase activity (Giaquinta 1979; Silvius and Snyder 1979). In addition, invertase activity in the alkaline range has also been detected in young sugar beet tap roots (Silvius and Snyder 1979). Despite the great economical importance of the partitioning of sugars in sugar beet, there is little knowledge of the genes encoding the enzymes of sucrose metabolism in this plant. Sucrose-phosphate synthase (Hesse et al. 1995) and sucrose synthase (Hesse and Willmitzer 1996) genes have been cloned and their expression analyzed. Regarding invertase genes, several partial cDNA sequences have been deposited in public databanks, but very little is known about invertase expression in sugar beet. Rosenkranz et al. (2001) described the cloning of partial cDNAs for two cell wall invertase and two vacuolar invertase genes from sugar beet and showed differential expression of these genes in wounded sugar beet roots. One of the cell wall invertase genes (CWI-1) was rapidly induced in wounded roots, whereas one of the vacuolar invertase genes (VI-1; Accession number AJ277457) showed a delayed induction, coincident with the increase in fructose and glucose in wounded roots. More recently, a cell wall invertase-like specific fructan 6-exohydrolase has been cloned and characterized from sugar beet (Van den Ende et al. 2003). Since sugar beet plants do not synthesize fructans, the function of this novel enzyme is not yet clear.

Here, we report on the cloning of three cDNAs encoding a vacuolar invertase and two extracellular invertases from sugar beet and the analysis of the pattern of expression of these genes in sugar beet plant organs during different stages of development. Our results show differential expression of acid invertase genes and a high expression of vacuolar invertase in petioles. Expression in petioles depends on the developmental stage and is highly influenced by light.

Materials and methods

Plant material and growth conditions

Sugar beet (*Beta vulgaris* L. cv. Claudia) seeds were provided by AIMCRA (Sevilla, Spain). Plants were grown at 20°C in vermiculite supplemented with nutrient solution NO_3-type with a photoperiod of 16 h light/8 h darkness, 70% humidity and a light intensity of 110 lx. After the desired time, plants were harvested and tap roots, shoot meristems, petioles and leaves were dissected, frozen in liquid nitrogen and kept at −80°C until required. The circadian rhythm was studied on 32-day-old plants taking samples at 4, 8 and 12 h after the start of the light period and 2 and 5 h after the start of the dark period. When the second day was maintained under continuous light or darkness, samples were collected at the same time intervals. For longer periods of growth, up to 75 days, plants were transferred to bigger size pots after 39 days.

Cloning of a cDNA for vacuolar invertase

Total RNA samples (1.2 μg) from different organs were reverse-transcribed in the presence of oligo-dT and 200 U of Reverse Transcriptase (Invitrogen). An aliquot of these reactions (5 μl) was then directly used as the template in a PCR reaction in the presence of 500 pmol each of the degenerative oligonucleotides *AI-3*, 5′-ACNGGNATGTGGGARTGY-3′ and *AI-4*, 5′-AYNGCYTTNGTNGGRTANA-3′, with N = A, C, G, T; R = A, G; Y = T, C. A 0.9-kb fragment was obtained, cloned and sequenced to confirm that it corresponded to vacuolar invertase. The cloning of the 5′ and 3′ cDNA ends was carried out by RACE (Rapid Amplification of cDNA ends) with the Marathon cDNA synthesis kit (Clontech), according to the manufacturer's instructions, with gene-specific primers *AIV-3* (5′-GGACTTGATACATCCACTAATG-3′) for the 3′-end and *AIV-1* (5′-CCTGTCATCATCCATGCTTGC-3′) for the 5′-end.

Cloning of cDNAs for two extracellular invertases

To obtain cDNAs for the two extracellular invertases from sugar beet, specific oligonucleotides for the 5′ and 3′ ends were designed for each gene, based on the sequences available in public databases under the accession numbers X81797 (BVBIN46) and AJ278531 (BVU278531) for BVInv-CW2 and BVInv-CW1, respectively. Poly(A)$^+$ RNA was isolated from shoot meristem with the Oligotex kit (Qiagen AS, Oslo, Norway) and 1.25 μg was used to construct a cDNA library

with the Marathon cDNA synthesis kit (Clontech), according to the manufacturer's instructions.

In the case of BVInv-CW2 both cDNA ends were obtained with the specific primers, BVEI2-2 (5′-AGCAACCACCCAATTCATAAG-3′) for the 5′-end and BVEI2-3 (5′-ATTATAAGGCTGAAAGAAATGGGTTT-3′) for the 3′-end. The BVInv-CW1 cDNA was completed at the 5′-end with the specific primer BVEI-1 (5′-GGCTGAACCTGACCAACTTCC-3′).

RNA isolation, northern blot and relative RT-PCR analysis

RNA was isolated with the guanidine hydrochloride method (Logemann et al. 1987), except that the RNA pellet was washed twice with 3 M sodium acetate and then with 70% (v/v) ethanol. For northern blot analysis, RNA samples (10 μg) were fractionated in formaldehyde agarose gels, transferred to Hybond–N membranes (Amersham Biosciences) and hybridized with ^{32} P-labeled vacuolar invertase partial cDNA (0.9-kb fragment described above) or SBSS 1 (Hesse and Willmitzer 1996) under standard conditions (Sambrook et al. 1989).

Relative RT-PCR analysis was performed as described elsewhere (Domínguez et al. 2002) with *AIV-5* (5′-TTTCAATGGAGTGTGGACTGGCT-3′) and *AIV-6* (5′-TACCCTCAGAGGTTAGCCAAGCAG-3′) as vacuolar invertase gene-specific primers, *SBSS-1* (5′-AATTCTTAGCGATGGCCCGTTT-3′) and *SBSS-2* (5′-CAGCAGTATCACCCCAGCCTCT-3′) as sucrose synthase gene-specific primers, *BVEI-5* (5′-GGTCCACCAGTATGGGGCCACTC-3′) and *BVEI-6* (5′-CCCATAATCATACCTCAAACTCGAGTC-3′) as extracellular invertase 1 (BVInv-CW1) gene-specific primers or *BVEI2-1* (5′-GTAAACTGGGTTCACCTTGAGC-3′) and *BVEI2-4* (5′-CAGTTGCTTCTACCTGAAACCC-3′) as extracellular invertase 2 (BVInv-CW2) gene-specific primers. The linear range of amplification was studied for each gene in order to establish the number of cycles of PCR to be used according to the manufacturer's instructions (Ambion, Austin, TX, USA). The proportion of 18S rRNA primers and competimers in each case was also determined as function of the abundance of each mRNA (2:8 for SBSS1, 1.5:8.5 for BVInv-V3 and 1:9 in all the other cases). Control reactions, using as template non-reverse-transcribed RNA, were performed to rule out possible amplification from contaminating genomic DNA. Cross-amplification of the different genes was ruled out by control reactions with different combinations of the PCR primers.

Invertase activity determination

The activity of invertases was measured as described previously (Roitsch et al. 1995). The amount of glucose liberated in the reaction was determined by the use of a glucose test kit (Roche, Indianapolis, IN, USA). In all cases, control reactions using the same volume of water instead of sucrose in the reaction mixture were performed. The concentration of protein in the samples was determined according to Bradford (1976) with the Bio-Rad kit. Invertase activity was determined in triplicate for each of the sample analyzed. In each condition analyzed, the sample consisted of petioles of at least six different plants. In the circadian experiments, two independent determinations, using two different pools of plants grown in the same conditions, were carried out obtaining the same results.

Sugar determination

The level of soluble sugars was determined as described previously (Balibrea et al. 2004) using a high-pressure liquid chromatography system coupled with pulsed amperometry detection (Dionex 4500; Dionex Softron, Germering, Germany). Sugar content for each individual sample was analyzed at least in duplicate. As previously described, the sample for analysis consisted of petioles of at least six different plants.

Results

Cloning of acid invertase genes from sugar beet

The cloning strategy to isolate cDNAs encoding acid invertases from sugar beet was based on RT-PCR. Initial amplification of a vacuolar invertase cDNA was carried out with a pair of degenerative primers designed in two well-conserved motifs of plant acid invertases (marked with arrows in Fig. 1) using as template RNA from different sugar beet plant organs. A 0.9-kb fragment was obtained showing high similarity with vacuolar invertases, in particular with cDNA VI-2, previously described from sugar beet (Rosenkranz et al. 2001). The corresponding 5′ and 3′-ends of the new vacuolar invertase cDNA, stated BVInv-V3, and cDNAs for two extracellular invertases, BVInv-CW1 and BVInv-CW2, with partial sequences already available in databases, were obtained by 5′ and 3′-RACE (rapid amplification of cDNA ends) using gene-specific primers.

BVInv-V3 cDNA (accession number AJ422051) contained a unique open reading frame flanked by 68 nucleotides at the 5′-UTR and 468 nucleotides at the 3′-UTR, including a poly-A tail. It encodes a deduced polypeptide of 675 residues with an expected molecular mass of 75.7 kDa and p *I* of 5.72, showing a level of similarity of 55–93% with vacuolar invertases from other plants available in public databases. BVInv-V3 contains a putative signal peptide of 59 residues at the N-terminus (marked with vertical arrow in Fig. 1). The cDNA for BVInv-CW1 (accession number AJ422052) contained a unique open reading frame flanked by 83 nucleotides at the 5′-UTR and seven nucleotides at the 3′-UTR. It

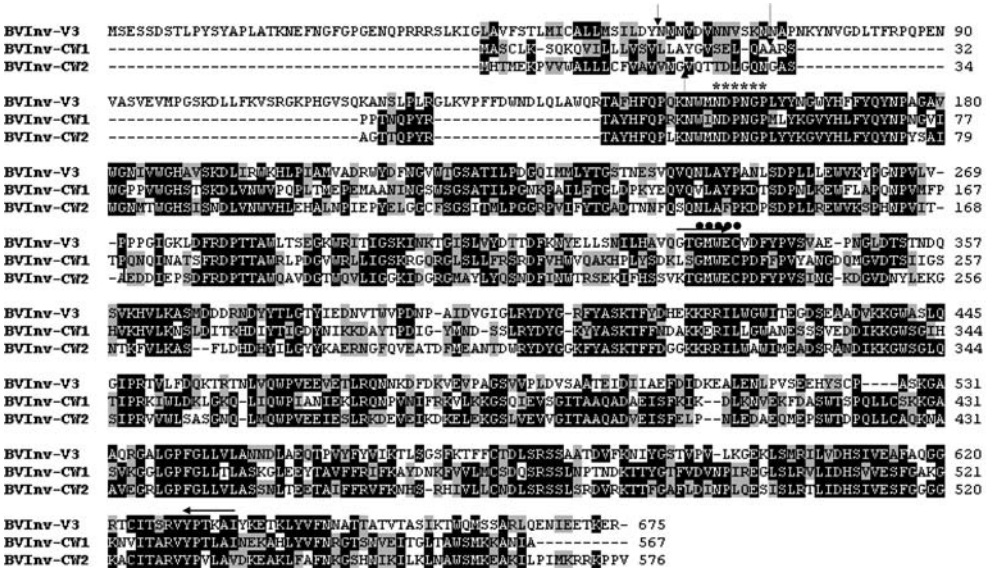

Fig. 1 Sequence alignment of the deduced proteins corresponding to sugar beet vacuolar invertase (BVInv-V3), extracellular invertase 1 (BVInv-CW1) and extracellular invertase 2 (BVInv-CW2) cDNAs. Sequences were aligned using the program BioEdit sequence alignment editor (Hall 1999). The *asterisks* and *black dots* mark motifs important for invertase catalysis. *Vertical arrows* mark the putative signal peptide cleavage sites (Neilsen et al. 1997) for each protein, determined by the use of ProScan tools (Combet et al. 2000). *Horizontal arrows* indicate the sequences for the design of degenerative oligonucleotides

encodes a deduced polypeptide of 576 residues with an expected molecular mass of 64 kDa and p *I* of 9.6. The cDNA for BVInv-CW2 (accession number AJ422053) contained a unique open reading frame, which encoded a deduced polypeptide of 576 residues with an expected molecular mass of 65 kDa and p *I* of 5.83, flanked by 38 nucleotides at the 5′-UTR and 368 nucleotides at the 3′-UTR, which included a poly-A tail. Both extracellular invertases contain putative signal peptides at the N-terminus (marked with vertical arrows in Fig. 1) of 29 and 22 residues for BVInv-CW1 and BVInv-CW2, respectively. A short hydrophobic C-terminal extension is present in BVInv-CW2 but absent in BVInv-CW1.

Figure 1 shows the sequence comparison of the polypeptides deduced from the three cDNAs. All of them present the motif NDPNGP (Fig. 1, asterisks) conserved in acid invertases and the motif WECV/PD (Fig. 1, dots) including a Cys residue important for invertase catalysis. The presence of a Val residue in this motif is characteristic of intracellular acid invertases, whereas in extracellular acid invertases the Val residue is replaced by Pro (Goetz and Roitsch 1999, 2000). Therefore, based on the features of the deduced polypeptides, we conclude that BVInv-V3 cDNA encodes a vacuolar acid invertase and BVInv-CW1 and BVInv-CW2 encode extracellular invertases.

Expression analysis of acid invertases in sugar beet plants

To address the contribution of the different sucrose-cleaving enzymes to sucrose metabolism in sugar beet plants, the pattern of expression of acid invertase genes was analyzed and compared with the pattern of expression of a previously reported sucrose synthase gene (SBSS1 gene, Hesse and Willmitzer 1996). The northern blot analysis, using gene-specific probes, did not detect extracellular invertase gene transcripts but detected vacuolar invertase transcripts exclusively in the leaf petiole of

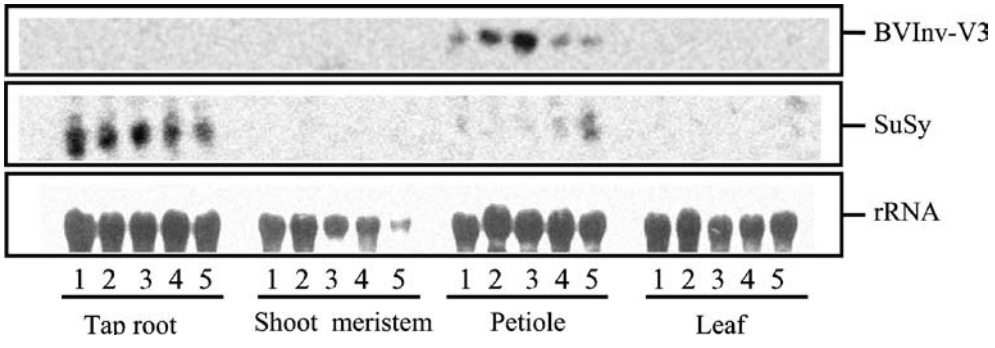

Fig. 2 Northern blot analysis of vacuolar invertase (BVInv-V3) and sucrose synthase (SuSy) transcripts in sugar beet organs. Sugar beet plants were grown in culture chambers for 32 days with a 16 h light/8 h dark cycle and then harvested at 4 h (1), 8 h (2), 12 h (3) after starting the light period and 2 h (4) and 5 h (5) after starting the dark period. At these time intervals leaves, petioles, shoot meristems and tap roots were dissected and RNA was isolated. RNA samples (10 μg) were fractionated on formaldehyde–agarose gels, blotted onto Hybond-N filters and hybridized to ^{32}P-labelled BVInv-V3 or sucrose synthase (SuSy) cDNA. Uniform loading was

32-day-old plants (Fig. 2). Vacuolar invertase transcripts increased during the light period (Fig. 2, lines 1–3) and then decreased during the dark period (Fig. 2, lines 4–5). In contrast, sucrose synthase transcripts were exclusively detected in tap roots and did not show any significant variation during the light or dark period (Fig. 2). It should be mentioned that the vacuolar invertase gene was expressed at a lower level (filters hybridized with the BVInv-V3 probe were exposed for 10 days) than sucrose synthase (filters exposed for 4 days).

To further analyze the pattern of expression of the extracellular invertase genes during plant development, the more sensitive technique of relative RT-PCR was used. Different tissues (tap roots, shoot meristems, petioles and leaves) were dissected from sugar beet plants grown for 32 and 75 days as representatives of juvenile and mature stages of development, respectively (Fig. 3a). In agreement with the northern blot data, vacuolar invertase transcripts were abundant in petioles of 32-day-old plants (Fig. 3b), whereas sucrose synthase transcripts showed a higher level in the tap root (Fig. 3c). The level of sucrose synthase did not show any significant variation in the two developmental stages analyzed (Fig. 3c); however, the amount of vacuolar invertase transcripts was clearly decreased in petioles from 75-day-old plants (Fig. 3b). Extracellular invertase genes showed a differential pattern of expression in sugar beet organs. BVInv-CW1 transcripts were more abundant in tap roots and decreased in 75-day-old plants (Fig. 3d), while BVInv-CW2 transcripts were more abundant in green tissues and the developmental stage had different influences depending on the organ (Fig. 3e).

Effect of the developmental stage on vacuolar invertase expression in petioles

The most relevant finding of the expression analysis shown above is the so far unknown specific expression of vacuolar invertase in petioles, which was higher in juvenile plants. The northern blot analysis (Fig. 2) was performed with a probe derived from the BVInv-V3 gene reported here, which probably cross-hybridized with other sugar beet vacuolar invertase genes (referred

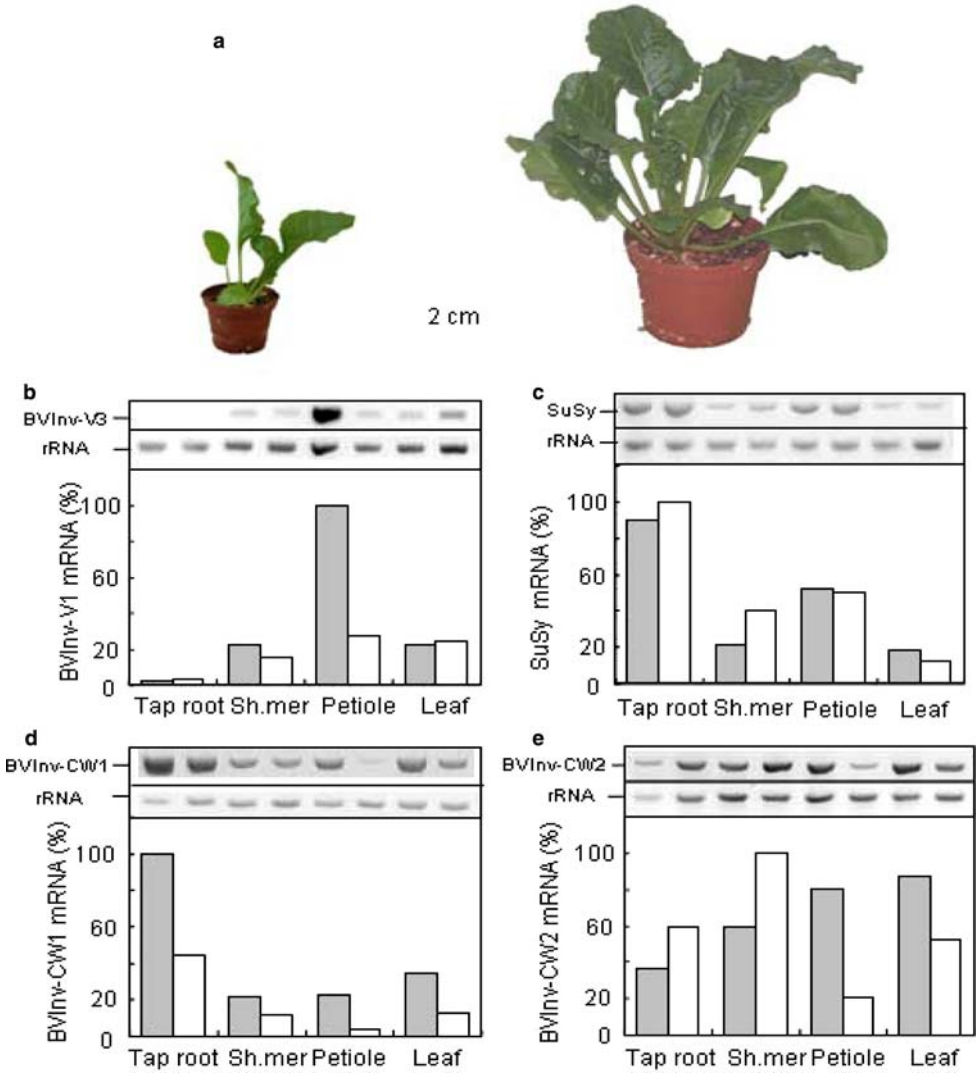

Fig. 3 Relative RT-PCR analysis of genes encoding sucrose-cleaving enzymes at different developmental stages. Sugar beet plants were grown in culture chambers for 32 days (**a**, left) or 75 days (**a**, right). Total RNA was isolated from dissected tap roots, shoot meristems (Sh mer), petioles or leaves and treated with DNase-I. After reverse transcription and RT-PCR in the presence of BVInv-V3 (**b**), SuSy (**c**), BVInv-CW1 (**d**) and BVInv-CW2 (**e**) gene-specific primers and 18S rRNA primers and competimers, the PCR products were fractionated on agarose gels. Bands were quantified using the program Scion Images. For each gene 100% was arbitrarily assigned to the maximum value, and the rest of values were represented as percentage of this value. Experiments were repeated at least three times and a representative result is shown. *Black bars*, 32-day-old plants; *open bars*, 75-day-old plants

to here as BVInv-V1 and BVInv-V2) previously reported (Rosenkranz et al. 2001). BVInv-V3 and BVInv-V2 share 98% identity at the nucleotide level and are probably alleles of the same gene. To analyze whether or not BVInv-V3 and the other vacuolar invertase gene, BVInv-V1, show differential expression in petioles, gene-specific primers were designed and the amount of both transcripts was analyzed by relative RT-PCR. Both vacuolar invertase genes showed a similar pattern of expression in petioles during plant development; a higher expression was observed in petioles of juvenile plants, which decreased with development to reach ~20% of the initial level (Fig. 4a). Vacuolar invertase activity in petioles (Fig. 4b) progressively decreased showing a good correlation with the level of transcripts.

Circadian oscillation of vacuolar invertase gene expression in petioles of sugar beet plants

The northern blot data raised the possibility that vacuolar invertase expression in petioles is light-dependent.

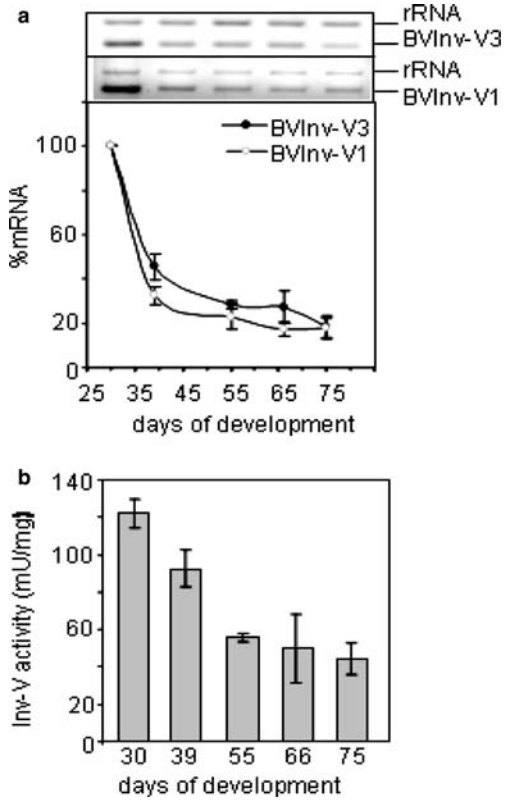

Fig. 4 Vacuolar invertase expression and activity during plant development. Sugar beet plants were grown in culture chambers for 32, 39, 55, 66 and 75 days. (a) Total RNA was isolated from dissected petioles and the content of transcripts of the two vacuolar invertase genes was studied by relative RT-PCR. (b) Vacuolar invertase activity in the soluble fraction of extracts corresponding to the same stages of development was determined at pH 4.5. At least three repetitions of the experiment were performed and a representative result is shown

The analysis of the level of BVInv-V3 and BVInv-V1 transcripts during a 24 h cycle (16 h light/8 h darkness) revealed that both transcripts showed a maximum accumulation at the middle of the light period (Fig. 5a). Vacuolar invertase activity in petioles varied also during the 24 h period, with the maximum activity delayed with respect to the mRNA peak (Fig. 5b). Extracellular and neutral invertase activities in petioles were also assayed. Variation of extracellular invertase was less pronounced than vacuolar invertase activity and the maximum of both acid invertases coincided (Fig. 5c); in contrast, neutral invertase activity was maximal at the end of the light period (Fig. 5d).

The expression pattern of the vacuolar invertase genes during a 24 h cycle suggested a circadian oscillation. To test this possibility we analyzed the variation of BVInv-V3 transcripts during a second 24 h period. Figure 6 shows that transcript oscillation was maintained during the second day, regardless of the incubation under normal light/dark cycle, continuous light or continuous darkness, thus suggesting that the expression of the vacuolar invertase gene is under circadian control in petioles of sugar beet plants.

Lastly, the content of sugars in 32-day-old sugar beet petioles was analyzed during a 24 h cycle. Glucose content was lowest at the end of the light period and then increased during the night (Fig. 7a), a pattern very similar to the fructose oscillation (Fig. 7b), though the content of glucose was much higher than the content of fructose. The increase in hexoses is coincident with the increase observed in invertase activity. The content of sucrose in petioles was very low and did not show any clear oscillation (Fig. 7c), showing a minimum at the end of the light period and the beginning of the dark period, again coincident with maximal neutral and acid invertase activities.

Discussion

Sucrose-cleaving enzymes play a central role in carbon metabolism and sugar-based signalling (Koch 2004). Most of the information on these enzymes has been obtained from the analysis of model plants; however, the knowledge of sucrose metabolism in economically important sucrose-producing crops is very limited. The aim of the study reported here was to analyze the possible function of acid invertases on sucrose metabolism in sugar beet, an economically important crop for sucrose production.

The three cDNAs reported here show sequence features of acid invertases. Based on the presence of Val or Pro residues in the conserved WECV/PD motif and the extension of 40 residues at the N-terminus, which is characteristic of vacuolar invertases, we deduced that BVInv-V3 cDNA encodes a vacuolar invertase whereas BVInv-CW1 and BVInv-CW2 encode extracellular invertases. Both extracellular invertases showed a level of similarity of 50% with each other (Fig. 1). The level of

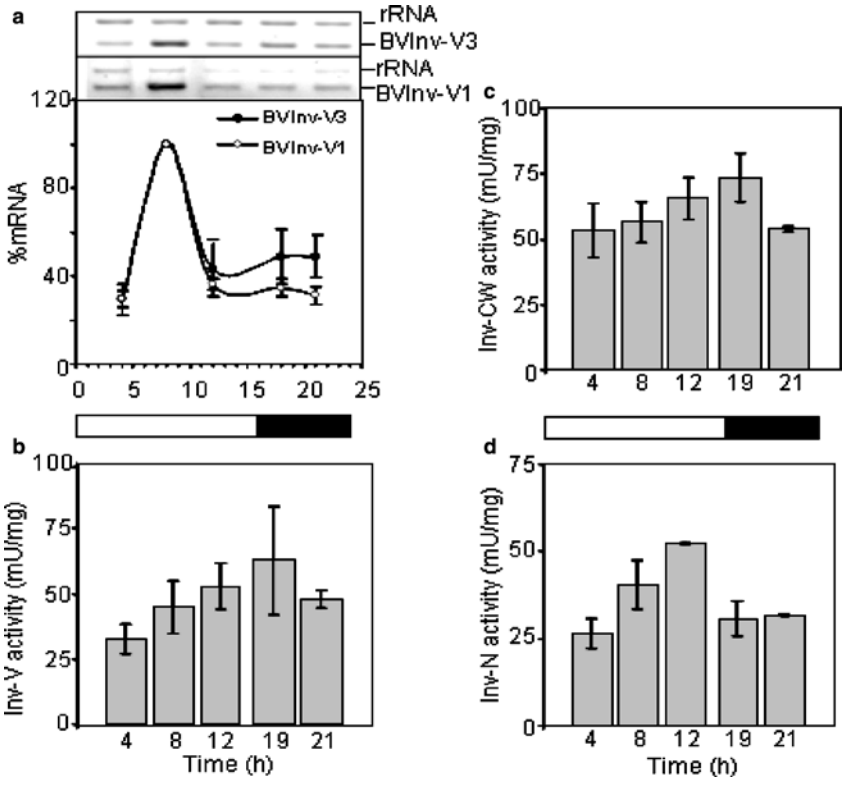

Fig. 5 Diurnal oscillation of vacuolar invertase expression and invertase activity in sugar beet petioles. Sugar beet plants were grown in culture chamber with a 16 h light/8 h dark cycle and then harvested at 4, 8 and 12 h after the onset of the subjective day and 2 and 5 h after the onset of the subjective night. **a** Total RNA was isolated from petioles and used in Relative RT-PCR reactions for analyzing the expression of BVInv-V3 and BVInv-V1 genes. Invertase activity was measured in the insoluble and soluble fractions of protein extracts corresponding to each time point. Vacuolar invertase activity **b** in the soluble fraction and extracellular invertase activity **c** in the insoluble fraction were determined at pH 4.5. Neutral invertase activity **d** in the soluble fraction was determined at pH 7.0. Representative results of at least three replicates are shown in the figure

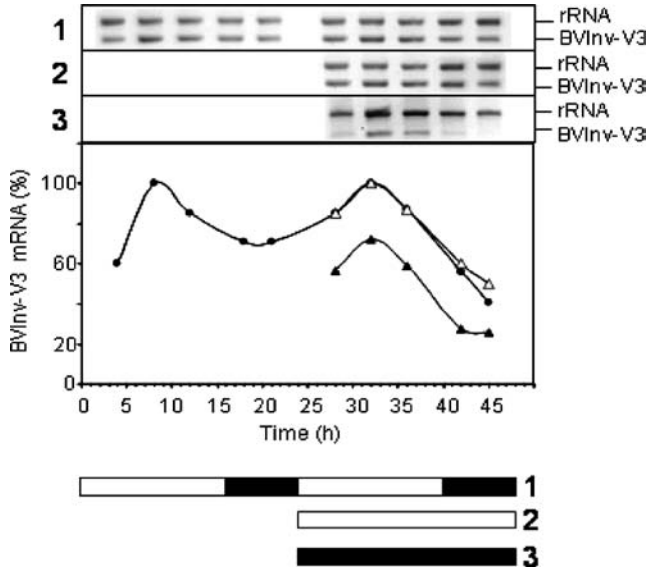

Fig. 6 Circadian expression of BVInv-V3 gene in petioles of sugar beet. Sugar beet plants were grown for 32 days in growth chambers with a 16 h light/8 h dark cycle. Three sets of plants were then taken and incubated for 2 days with the same cycle (1, *black dots*), with continuous light during the second day (2, *open triangles*) or with continuous darkness during the second day (3, *black triangles*), as indicated at the bottom. Total RNA was isolated from dissected petioles and treated with DNase-I. After reverse transcription and Relative RT-PCR in the presence of BVInv-V3 gene-specific primers and 18S rRNA primers and competimers, the PCR products were fractionated on agarose gels. Bands were quantified using the program Scion Images. The maximum value was arbitrarily assigned 100% and the rest of values were represented as percentage of this value

similarity with extracellular invertases from other plants available in public databases was higher (67–91% similarity for BVInv-CW1 and 63–72% for BVInv-CW2). BVInv-CW1 deduced polypeptide, like most of the extracellular invertases described so far, has a basic pI. In contrast, the pI of the BVInv-CW2 deduced polypeptide is acidic, hence suggesting that it might be a new form of cell wall invertase present in a free form in the apoplast as has already been described for INCW4 from maize (Kim et al. 2000), CIN3 from *Chenopodium rubrum* (Ehness and Roitsch 1997) and a cell wall invertase from papaya fruits (Zhou et al. 2003; Roitsch and González 2004). Interestingly, both BVInv-CW2 and INCW4 are constitutively expressed in all vegetative tissues tested (Kim et al. 2000). Therefore, based on the features of the deduced polypeptides, we conclude that both cDNA clones described here encode extracellular acid invertases, but only BVInv-CW1 is a cell wall bound invertase.

The expression analysis of genes encoding sucrose-cleaving enzymes, acid invertases and sucrose synthase (Hesse and Willmitzer 1996) revealed a rather complex pattern in sugar beet plants. The predominant sucrose-cleaving gene in tap roots, the sucrose-storing organ, is sucrose synthase. Of the two extracellular invertase genes reported here (BVInv-CW1 and BVInv-CW2), which show differential expression in sugar beet organs, BVInv-CW1 is almost exclusively expressed in roots, though at lower level than sucrose synthase. These results suggest that sucrose metabolism in root cells depends on sucrose synthase, in agreement with previous

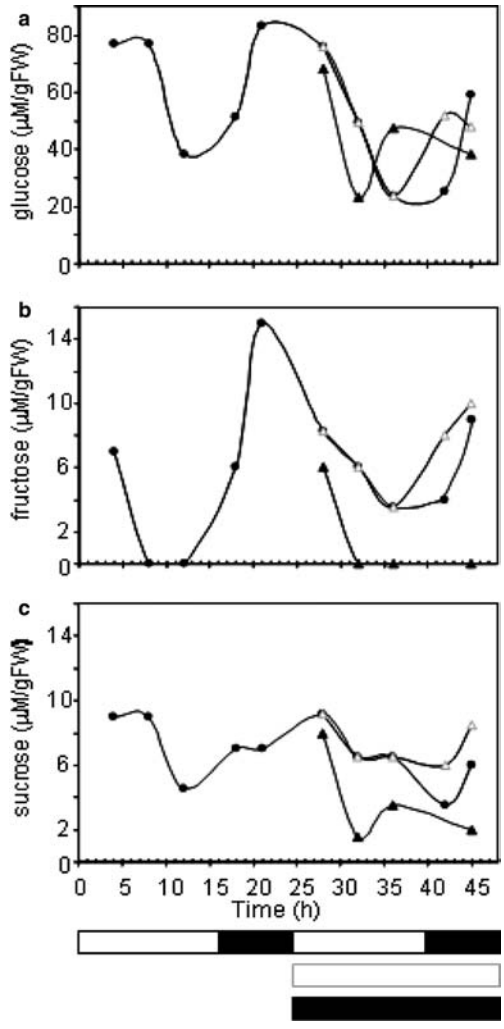

Fig. 7 Diurnal oscillation of soluble sugar content in petioles of sugar beet. Sugar beet plants were grown as described in Fig. 6 and incubated for 2 days with the same cycle (*black dots*), with continuous light during the second day (*open triangles*) or with continuous darkness during the second day (*black triangles*), as indicated at the bottom. The content of glucose (**a**), fructose (**b**) and sucrose (**c**) were determined in the petioles of plants harvested at the indicated time points. Determinations were performed on three different sets of plants and a representative result is shown

reports describing an increase of sucrose synthase activity and a decrease of invertase activity in sugar beet roots as sucrose storage proceeds (Giaquinta 1979; Silvius and Snyder 1979). In contrast, BVInv-CW1 gene, which probably encodes a cell wall invertase, might be involved in specific functions. The occurrence of acid invertase activity associated to the cell wall in sugar beet tap roots has been suggested by previous studies (Leigh et al. 1979). Since antisense suppression of extracellular invertase expression in carrot tap roots produced smaller roots with lower level of carbohydrates (Tang et al. 1999), a likely possibility is that BVInv-CW1 gene is involved in the regulation of sink strength in sugar beet tap roots in early stages of development.

Vacuolar invertase transcripts were not detected in tap roots even with the very sensitive RT-PCR approach, a result that suggests that vacuolar invertase does not play any role in the sucrose-storing tap root at least under normal growth conditions. This result is in agreement with the described decrease of soluble invertase activity in tap roots during sucrose storage, since the stages analyzed in this study correspond to active sugar storage in the root (Giaquinta 1979). The decrease of invertase activity along the development of sugar beet tap root has been hypothesized to be due to the formation of an invertase inhibitor (Giaquinta 1979). The results described here do not allow to rule out this possibility, but suggest that the low invertase activity is at least in part due to a non-detectable expression of vacuolar invertase genes. Despite the low-level expression of vacuolar invertase in roots, physical dissection increases vacuolar invertase activity (Giaquinta 1977) and abiotic stress, such as wounding, triggers the induction of a vacuolar invertase gene (Rosenkranz et al. 2001), which suggests an important role of vacuolar invertase in response to environmental stimuli.

A remarkable result of the expression analysis of sucrose-cleaving genes in sugar beet is the so far unknown specific expression of the vacuolar invertase gene (BVInv-V3) in petioles. Here, we provide evidence of two major factors involved in the regulation of vacuolar invertase expression in petioles: the developmental stage of the plant and the circadian oscillation.

The two vacuolar invertase genes from sugar beet analyzed here, BVInv-V3 and BVInv-V1, show a similar pattern of expression in petioles of growing plants, transcripts content being highest in the most juvenile stage analyzed (32-day-old plants). This pattern of expression shows a good correlation with vacuolar invertase activity, hence suggesting a close relationship of active growth and vacuolar invertase. This is the case of actively growing roots of carrot (Ricardo and Ap Rees 1970) or bean stems (Morris and Arthur 1985), which show a high acid invertase activity. A primary role of vacuolar invertase in actively growing tissues is to provide hexoses to support growth (Tymowska-Lalanne and Kreis 1998; Winter and Huber 2000). In addition, the hydrolysis of sucrose to glucose and fructose doubles the osmotic pressure aiding elongation of tissues by cell expansion. A possible role for vacuolar invertase in cell elongation was suggested by the expression of the corresponding genes in developing petioles and primary roots of carrot plants (Sturm et al. 1995). Furthermore, this pattern of expression is in agreement with the profile of contribution of sucrose-cleaving enzymes during development, as summarized by Koch (2004), in which vacuolar invertases are the first sucrose-cleaving enzymes to be expressed. The observation that sucrose is not hydrolyzed prior to phloem loading or during transit to the storing root (Giaquinta 1977) seems to contradict the results described here. Nevertheless, these observations were obtained with 8 to 10-week-old plants. The

decrease of vacuolar invertase expression after 75 days of development agrees with their result, reinforcing the hypothesis that vacuolar invertase expression in petioles of young plants is associated to active growth.

The second relevant feature of vacuolar invertase expression in petioles is that it shows circadian oscillation. Recently, a model of circadian regulation of cell elongation in *Arabidopsis* has been postulated by Harmer et al. (2000), based on the observation that a number of genes implicated in cell elongation, including auxin efflux carriers, are circadian-regulated and show maximum expression at the end of the subjective day, at the time of most rapid cell elongation in the hypocotyls of young seedlings. This model supports the hypothesis that vacuolar invertase in sugar beet petioles is associated with active growth. A light–dark oscillation of vacuolar invertase activity and transcript level has been reported in maize drought-stressed shoots (Kim et al. 2000), although the oscillation was not completely characterized. Our results show that the level of sucrose in petioles is kept low and does show a low oscillation, therefore, it is unlikely that sucrose itself exerts any effect on the regulation of the vacuolar invertase genes. In contrast to sucrose, both glucose and fructose showed oscillation, which paralleled the activity of vacuolar invertase. However, the content of fructose was much lower than glucose, which suggests a differential uptake and metabolism of both sugars by petiole cells. In agreement with this result, it has been shown that supply of sucrose to tomato suspension culture cells results in a differential accumulation of glucose and fructose in the medium (Sinha et al. 2002). This is another evidence of the important function of vacuolar invertase in sucrose cleavage in petioles. An additional consequence of the predominant expression of vacuolar invertase genes, and one of the extracellular invertases, is that the level of sucrose is low, as mentioned above, and thus the sink strength of this tissue is high, facilitating sucrose export from the leaves.

It has been described that sucrose synthase expression in other sugar beet organs is not affected by the presence of sugars (Hesse and Willmitzer 1996). In contrast, invertases are modulated by sugars in maize although with a complex pattern since *Ivr2* gene is upregulated by increasing carbohydrate supply, whereas *Ivr1* gene is repressed by sugars and upregulated by carbohydrate depletion (Xu et al. 1996). Also in *C. rubrum* suspension-culture cells, the induction of an apoplastic invertase by D-glucose has been reported, whereas the activities of neutral and acidic intracellular invertases were not affected (Roitsch et al. 1995).

To date, the major role assigned to vacuolar invertases is to be involved in the response of the plant to drought stress (Pelleschi et al. 1999; Kim et al. 2000; Andersen et al. 2002) or wounding (Rosenkranz et al. 2001). Our results showing the specific expression of vacuolar invertase in petioles and its dependence of the developmental stage and circadian rhythm suggest an important role of this vacuolar invertase gene in plant development and photoassimilate partitioning in sugar beet.

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